

Association between *ihha* and *shha* gene downregulation and swim bladder inflation impairment in zebrafish exposed to the combination of morphine and disulfiram*

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Recent evidence suggests that disulfiram may serve as a useful adjunct in chronic morphine treatment by mitigating opioid-induced hyperalgesia, withdrawal, and dependence. However, exposure to disulfiram produces abnormalities in swim bladder inflation in zebrafish, which points to the possibility of interference with Hedgehog signaling - a key regulator of swim bladder formation. Therefore, this study aimed to find the association between the expression of markers present in the

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developing zebrafish swim bladder (*sox2*, *fgf10a*, *pbx1a*, *acta2*, *anxa5*) and the Hedgehog pathway genes (*ptch1*, *ptch2*, *smo*, *ihha*, and *shha*) in zebrafish embryos treated with the morphine-disulfiram combination. For this purpose, zebrafish were treated for 54 or 117h with disulfiram alone or in combination with morphine. Swim bladder inflation was assessed under a light microscope, and gene expression was quantified with RT-qPCR assay using SYBRGreen dye. Morphine alone upregulated *ptch2*, *smo*, and all of the other markers present in the swim bladder except for the *ihha* gene; while disulfiram alone downregulated all Hh pathway genes in the zebrafish embryos along with *fgf10a* and *pbx1a* markers. When combined with morphine, the expression of *shha* and *ihha* further decreased, while *ptch1* increased considerably. An upregulation of *sox2* and *fgf10a* swim bladder markers was also present. Our investigation has shown that the synergistic disruption in swim bladder inflation in zebrafish treated with the morphine-disulfiram combination could be associated with *ihha* and *shha* downregulation, triggering a compensatory upregulation of *acta2* and *anxa5* expression.

KEY WORDS: morphine / disulfiram / swim bladder inflation /
zebrafish / Hedgehog pathway

The Hedgehog (Hh) signaling pathway plays a pivotal role in embryonic development in vertebrates. Within the Hh family, two signaling proteins - Sonic Hedgehog (Shha) and Indian Hedgehog (Ihha) - are most widely expressed. Shha signaling is mostly responsible for proper neural tube patterning and body segmentation [Kahane *et al.* 2019], neuronal specification [Memi *et al.* 2018], and lung [Pepicelli *et al.* 1998a] and muscle morphogenesis [Jia *et al.* 2018]. The *ihha* ligand governs bone development in the limbs and craniofacial region [Amano *et al.* 2020, St-Jacques *et al.* 1999] and angiogenesis [Colnot *et al.* 2005]. Dysfunction in the Hh pathway leads to developmental abnormalities including polydactyly, facial malformations such as cleft lip and palate, and holoprosencephaly [Abramyan 2019]. Hedgehog signaling has also been implicated in tumorigenesis and metastasis in adults [Liao *et al.* 2009, Tostar *et al.* 2010, Yoo *et al.* 2011].

Expression of *shha* and *ihha* genes - patched 1 (*ptch1*) and patched 2 (*ptch2*) - takes place in the epithelium, while the coding for their receptors occurs in the mesenchyme. These two genes are crucial to the organization and inflation of the zebrafish swim bladder [Winata *et al.* 2009a]. This organ is used for buoyancy maintenance in teleost fish and bears evolutionary relatedness to the terrestrial vertebrate lung [Zheng *et al.* 2011]. Toxicology studies have revealed that many exogenous factors disturb Hh signaling and swim bladder inflation in zebrafish, including heavy metals like copper and cadmium [Xu *et al.* 2017, Zhang *et al.* 2022], insecticides [Bao *et al.* 2025] and even commonly used pharmaceuticals [He *et al.* 2025, Pandelides *et al.* 2021]. Recently, disulfiram has received increased attention regarding potential utilization beyond its current common use in treatment of alcohol use disorder and cocaine addiction. Many studies claim that disulfiram could be repurposed as an adjuvant for viral, microbial, or protozoal infections [Chen *et al.* 2022, Meneguello *et al.* 2022, Sharma *et al.* 2022], as well as inflammatory diseases [Guo *et al.* 2021] or antineoplastic therapy [Zeng *et al.* 2024].

Of note, disulfiram also surfaced as a potential drug of interest in the treatment of central nervous system disorders like Alzheimer's disease, as well as opioid-induced

hyperalgesia, tolerance, and withdrawal [de Corde-Skurska *et al.* 2021, de Cordé *et al.* 2018, Hosobuchi *et al.* 1977, Reinhardt *et al.* 2018]. Repurposing disulfiram as an adjunct drug for the mitigation of opioid-induced pain sensitization is of particular relevance, as only few pharmaceutical options exist for its management in chronic pain patients. Additionally, adjuvant therapies have been suggested as a potent opioid sparing strategy, although apart from disulfiram, no registered drug has been shown to preemptively suppress hyperalgesia thus far [Guichard *et al.* 2021].

However, dithiocarbamates such as disulfiram were also shown to cause developmental abnormalities in zebrafish involving, but not limited to, loss of pigmentation and craniofacial and tail malformations resulting from disturbances in cartilage ossification [van Boxtel *et al.* 2010]. The teratogenic potential of disulfiram is so far poorly understood. Available reports point to its capacity to generate reactive oxygen species (ROS) by inhibiting a variety of enzymes, either by chelating copper ions or direct inhibition by means of disulfide bridge formation in the active site [Yuan *et al.* 2023]. Dithiocarbamates were also shown to cause downregulation of transcription factors important for both craniofacial and swim bladder development [Desai *et al.* 2011, Winata 2009a]. Of note, our latest investigation demonstrated that disulfiram synergistically impairs swim bladder inflation when combined with morphine [Zelazewska *et al.* 2025a]. However, the mechanistic underpinnings of this phenomenon have not yet been investigated. Therefore, the present study was designed to evaluate how exposure of zebrafish embryos to disulfiram and morphine affect the expression of key swim bladder development markers *sox2*, *fgf10a*, *pbx1a*, *acta2* and *anxa5*. It was also investigated whether their expression was related to the expression of *ptch1* and *ptch2*, as well as *smo*, *ihha* and *shha* genes of the Hh pathway.

Material and methods

Chemicals and kits

Disulfiram and morphine hydrochloride were kindly donated by Polpharma (Starogard Gdański, Poland). The PureLink™ RNA Mini Kit, PureLink™ DNase Set, and High-Capacity cDNA Reverse Transcription Kit were purchased from ThermoFisher Scientific (Waltham, MA, USA). Media components for zebrafish fertilization and maintenance were purchased from Sigma-Aldrich (St. Louis, MO, USA). GoTaq® qPCR Master Mix was obtained from Promega (Madison, WI, USA). Primers for qPCR were designed with PrimerBLAST (<https://www.ncbi.nlm.nih.gov/tools/primer-blast/>) and Oligonucleotide Properties Calculator (<http://www.basic.northwestern.edu/biotools/oligocalc.html>). Primer sequences were synthesized by Genomed (Warsaw, Poland). Primer sequences are listed in Supplementary data, Table S1.

Zebrafish maintenance and treatment

Zebrafish (*Danio rerio*) embryos of the AB background were purchased from the Centre of Experimental Medicine, Medical University of Lublin, Poland. Larvae were

kept in the incubator, with a temperature adjusted to $28.5 \pm 0.5^\circ\text{C}$ and a 14h light:10h dark light cycle. Zebrafish were bred in the E3 medium (17.4mM NaCl, 0.21mM KCl, 0.12mM MgSO_4 , 0.18mM $\text{Ca}(\text{NO}_3)_2$, 1.5mM HEPES, 0.6 μM methylene blue, pH 7.6). In agreement with the current European Union legislation, ethical permission to conduct experiments on larvae is not required up to 120 hours post-fertilization (hpf). However, we made all efforts necessary to avoid animal suffering in compliance with the National Institute of Health Guidelines for the Care and Use of Laboratory Animals and the European Community Council Directive of November 2010 for Care and Use of Laboratory Animals (Directive 2010/63/EU), honoring the ARRIVE guidelines.

One hour after fertilization, embryos were carefully selected for experiments. Perfectly transparent, viable, and round embryos were transferred to the wells of a 12-well plate with disulfiram (0.1-1 μM) and morphine (10 μM), alone or in combination. Drugs were dissolved in DMSO, so that the final DMSO concentration in the medium was 0.5%. The control group was treated with 0.5% DMSO, and drug solutions were replaced daily. In experiments measuring early gene expression, embryos were treated for 54h. Subchronic experiments employed the analysis of late gene expression (*acta2* and *anxa5a*) and involved a 117h treatment regimen. The larvae were examined for general morphological traits under an Olympus stereomicroscope (body curvature, yolk sac necrosis, heart edema, changes in pigmentation, jaw development, and swim bladder inflation) at 118h.

RNA extraction

Total RNA was isolated from either zebrafish embryos or larvae with the PureLink™ RNA Mini Kit (Waltham, MA, USA) according to the manufacturer's instructions. Embryos were collected from 4 separate wells per treatment group. Within the treatment groups, larvae were divided into swim bladder positive (+) or negative (-). Eleven larvae were collected from each 3-4 separate wells. Due to the inability to collect enough swim bladder positive (+) or negative (-) larvae treated with 1 μM of disulfiram alone or in combination with morphine, the expression analysis was not performed on those groups. Samples were then mixed with 600 μL of Lysis Buffer containing 2-mercaptoethanol and homogenized on ice with a hand-held motor-driven Tissue Grinding Tool (EurX, Gdańsk, Poland). The homogenate was centrifuged for 2min. at $12,000 \times g$. Next, the supernatant was transferred to a clean tube and samples were mixed with 600 μL 70% ethanol and deposited onto a RNA-binding spin column. Columns were centrifuged at $12,000 \times g$ for 15s. 350 μL of Wash Buffer I was added to the column and centrifuged at $12,000 \times g$ for 15s. Then, 80 μL of PureLink™ DNase Mixture was added onto the spin column and incubated for 15min. at room temperature (RT). 350 μL of Wash Buffer I was added to the spin column and samples were then centrifuged at $12,000 \times g$ for 15s. The spin column was washed with 500 μL of Wash Buffer II containing ethanol and centrifuged at $12,000 \times g$ for 15s. The washing step was repeated one more time. The columns were centrifuged again at $12,000 \times g$ for 1min. to dry the column. Total RNA was eluted by adding 30 μL of RNase-free water,

incubating the sample for 1 min. at RT and centrifugation at $12,000 \times g$ for 2 min. Total RNA concentration and purity were determined on the UV-Vis NanoDrop 2000c/2000 spectrophotometer. The RNA samples with a 260/280 ratio between 1.95-2.2 were considered pure. RNA was stored at -80°C until reverse transcription PCR was performed.

RT-qPCR analysis of gene expression

Reverse transcription was carried out with the High-Capacity cDNA Reverse Transcription Kit (ThermoFisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions. 100 ng of total RNA was used for the reaction. Samples were loaded to the Biometra T Personal (Analytik Jena, Germany) thermocycler and the following reaction conditions were applied: 10 min. at 25°C , 120 min. at 37°C and 5 min. at 85°C . The reaction mixture was then cooled to 4°C . cDNA was stored at -20°C until qPCR was performed. Quantitative PCR was performed using an Illumina Eco real-time PCR system (Illumina Inc. San Diego, USA) with BRYTGreen as the fluorescent dye. Samples contained 10 μl of GoTaq qPCR Master Mix, 1 μl of each primer, 4 μl H_2O , and 40 ng of cDNA template in a final reaction volume of 20 μl . Ribosomal RNA (*18S rRNA*) [Gao *et al.* 2024] and *elongation factor 1-alpha 1-like 1* (*elfa*) [McCurley *et al.* 2008] were used as reference genes. The reaction parameters were set to 95°C for 10 min., followed by 40 cycles of the following: 95°C for how long? and 61°C for 1.30 min. The efficiency of the qPCR reaction was within the acceptable range of 90-110%, and a melting curve was included after each reaction run to confirm reaction specificity.

Statistical analysis

Expression data was presented as fold change in relation to control $\pm$ standard error of the mean (SEM). Data from the swim bladder analysis was expressed as % of the values obtained in the 0.5% DMSO group. Graphs were generated with GraphPad Prism 5.03 for Windows (GraphPad Software, Boston, Massachusetts USA, www.graphpad.com). Raw data was analyzed using STATISTICA v.13 (TIBCO Software Inc., CA, USA). The Shapiro-Wilk test was used to check for Gaussian distribution of data and the Leven's test was applied to check for equal variances. Parametric data was processed with one-way (with treatment as the main factor) or two-way ANOVA (with treatment and swim bladder status as factors). The Duncan test was used for post-hoc group comparisons. Non-parametric comparisons of medians were made with the use of the Kruskal-Wallis test. Pearson's chi-square test was used to process swim bladder formation data, and Pearson's correlation analysis was used to measure linear correlation between data sets. Drug synergy was determined with the Bliss Independence Model (BSS) [Zheng *et al.* 2022] provided by the online Synergy Finder Plus software. The threshold for statistical significance was set to $p \leq 0.05$.

Results and discussion

Disulfiram downregulates the expression of Ptch receptor ligands *shha* and *ihha* in zebrafish embryos

As shown in Figure 1, disulfiram alone (0.1-1 μ M) and in combination with morphine (10 μ M), decreased mRNA levels of Ptch1 and Ptch2 receptor ligands *shha* [$(F_{5,18} = 17, p < 0.001)$] and *ihha* [$(F_{5,24} = 54.5, p < 0.001)$]. Disulfiram at 0.1 μ M did not have any significant effect on *shha* gene expression, while at a higher 1 μ M concentration, mRNA levels were decreased below detection levels. On the other hand, disulfiram dose-dependently decreased *ihha* mRNA levels by 23% at 0.1 μ M and 51.2% at 1 μ M ($p < 0.001$). Morphine at a 10 μ M concentration completely abolished *shha* expression while having no effect on *ihha* mRNA levels. However, when morphine was combined with disulfiram at either 0.1 μ M or 1 μ M concentrations, a 52.4% ($p < 0.001$) and 64.4% ($p < 0.001$) decrease in *ihha* expression was observed in comparison with morphine-treated embryos. The *ihha* gene expression in the MRF + DSF0.1 μ M treatment group was also 31.7% lower when compared with disulfiram at 0.1 μ M ($p < 0.001$). A similar pattern was observed for the *shha* gene expression, where mRNA levels in the morphine + disulfiram 0.1 μ M treatment group were reduced by 87% ($p < 0.001$).

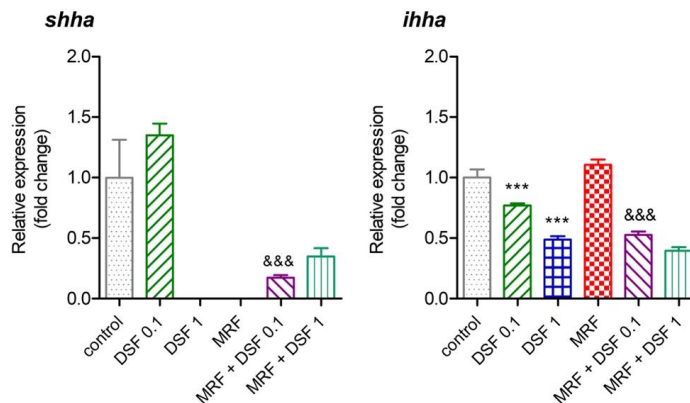


Fig. 1. mRNA levels of the Hedgehog pathway (Hh) genes - *shha* (Sonic Hedgehog) and *ihha* (Indian hedgehog) - in zebrafish embryos treated with disulfiram (DSF, 0.1-1 μ M), morphine (MRF, 10 μ M) and their combination (MRF + DSF) for 54h (n=4). *Shha* expression in the MRF+DSF1 groups was below the detection limit. *** $p < 0.001$ (vs. 0.5% DMSO control); &&& $p < 0.001$ (DSF0.1 vs. MRF+DSF0.1) (one-way ANOVA, Duncan's test).

The expression of the *ptch1* [$(F_{5,18} = 6.95, p < 0.001)$], *ptch2* [$(F_{5,24} = 8.95, p < 0.001)$] and *smo* [$(F_{5,18} = 19.5, p < 0.001)$] were also affected by morphine and disulfiram treatment (Fig. 2). Disulfiram at 0.1 μ M did not have any significant effect on *ptch1* gene expression, while it decreased *ptch2* and *smo* mRNA levels by 45% ($p < 0.05$) and 41.8% ($p < 0.001$) when compared with control embryos. The higher concentration of disulfiram (1 μ M) decreased mRNA levels of *ptch1*, *ptch2* and *smo* genes by 87.5%

($p < 0.05$), 47.1% ($p < 0.05$) and 51.2% ($p < 0.001$), respectively. Moreover, a significant positive correlation was detected between the *ptch2* and *ihha* ($r = 0.89$, $p = 0.04$) and *smo* ($r = 0.88$, $p = 0.047$) mRNA levels in embryos treated with $1 \mu\text{M}$ of disulfiram. Of note, *ptch1* expression decreased by 77.3% ($p < 0.05$) following morphine exposure, while the levels of *ptch2* and *smo* were only moderately increased by 49.8% ($p < 0.05$) and 30% ($p < 0.001$), respectively.

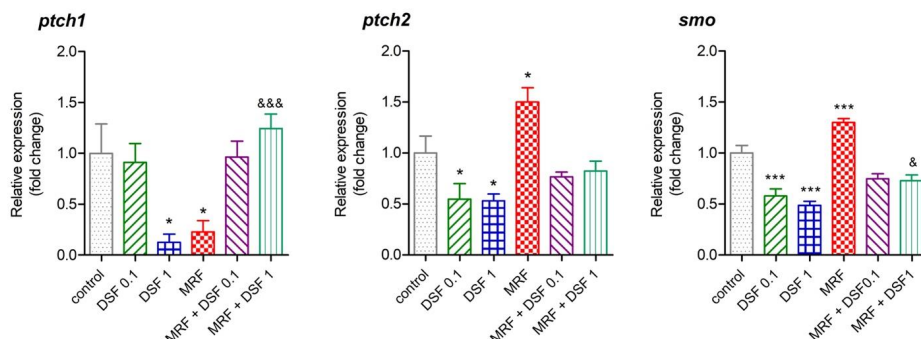


Fig. 2. mRNA levels of the early mesenchymal Hedgehog pathway (Hh) genes - *ptch1*, *ptch2* and *smo* - in zebrafish embryos treated with disulfiram (DSF, 0.1- $1 \mu\text{M}$), morphine (MRF, $10 \mu\text{M}$) and their combination (MRF + DSF) for 54h ($n = 4$). * $p < 0.05$, *** $p < 0.001$ (vs. 0.5% DMSO control); & $p < 0.05$, && $p < 0.001$ (DSF1 vs. MRF+DSF1) (one-way ANOVA, Duncan's test).

A combination of disulfiram (0.1- $1 \mu\text{M}$) and morphine restored *ptch1* expression when compared with disulfiram and morphine alone ($p < 0.01$). Additionally, a significant, positive correlation was detected between *ptch1* and *shha* gene expression (MRF+DSF1: $r = 0.96$, $p = 0.010$), while a tendency for a negative correlation was detected for the MRF+DSF0.1 group ($r = -0.85$, $p = 0.056$). A similar effect was observed for *ptch2* and *smo* gene expression, where disulfiram significantly decreased morphine-induced upregulation of *ptch2* to the levels seen in controls or below control levels for the *smo* gene ($p < 0.05$).

Disulfiram downregulates swim bladder development marker expression in zebrafish embryos

As presented in Figure 3, treatment with disulfiram, morphine, and their combination affected the swim bladder expression of *sox2* in the epithelium [$(F_{5,24} = 41.5, p < 0.001)$], *fgf10a* expression in the mesenchyme [$(F_{5,24} = 24.0, p < 0.001)$], and *pbx1a* expression in all three embryonic swim bladder layers [$(F_{5,24} = 19.6, p = 0.0015)$]. Disulfiram alone at $1 \mu\text{M}$ did not affect *sox2* expression levels; however, a significant negative correlation was detected between *sox2* and *ptch1* expression ($r = -0.90$, $p = 0.038$), as well as a positive correlation with *ptch2* ($r = 0.90$, $p = 0.035$). The expression levels of the *fgf10a* and *pbx1a* markers at a $0.1 \mu\text{M}$ disulfiram concentration were decreased by 43.3% ($p < 0.05$) and 62.4% ($p < 0.01$), respectively. A similar level of *pbx1a* gene downregulation was also observed for the $1 \mu\text{M}$ concentration (52.6%,

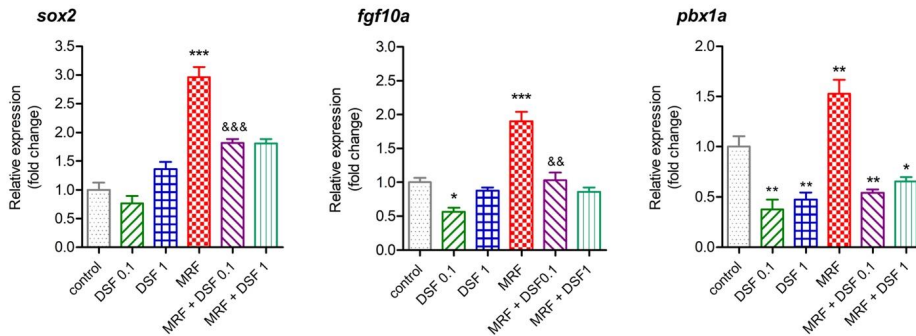


Fig. 3. mRNA levels of early swim bladder development genes *sox2* (epithelial marker), *fgf10a* (mesenchymal marker), and *pbx1a* (epithelial, mesenchymal and mesothelial marker) in zebrafish embryos treated with disulfiram (DSF, 0.1–1 μ M), morphine (MRF, 10 μ M) and their combination (MRF + DSF) for 54h (n=4). * p <0.05; ** p <0.01; *** p <0.001 (vs. DMSO control); && p <0.01 (DSF vs. MRF+DSF) (*sox2* and *fgf10a*: one-way ANOVA, Duncan's test; *pbx1a*: Kruskal-Wallis test).

p <0.01). Additionally, the downregulation of *pbx1a* was positively correlated with the expression of *ptch2* ($r = 0.97$, $p = 0.035$) and *smo* ($r = 0.97$, $p = 0.005$) genes.

Morphine substantially upregulated *sox2*, *fgf10a*, and *pbx1a* mRNA levels 196%, 90% (p <0.001), and 52% (p <0.01), respectively. A positive correlation was detected between *pbx1a* and *ptch2* expression ($r = 0.95$, $p = 0.013$). Disulfiram significantly decreased morphine-induced upregulation of *sox2* (p <0.001) and *fgf10a* genes (p <0.01) to or below control levels, as seen also with the *pbx1a* gene (p <0.01 for the MRF+DSF0.1 group and p <0.05 for the MRF+DSF1 group). Additionally, a positive correlation existed between the *sox2* and *smo* gene expression ($r = 0.96$, $p = 0.013$) at the 0.1 μ M disulfiram concentration.

The disulfiram-morphine combination impairs swim bladder development

As illustrated in Table 1, a significant reduction in the number of larvae with a properly inflated swim bladder was observed only for 10 μ M of morphine ($\chi^2 = 6.18$, $p = 0.013$). At the 0.1 μ M disulfiram concentration, 76.9% of larvae exhibited proper swim bladder inflation, representing a significant reduction compared with the control group ($\chi^2 = 4.17$, $p = 0.041$). Furthermore, co-exposure to 0.1 μ M disulfiram significantly reduced swim bladder inflation in larvae treated with 1 ($\chi^2 = 6.01$, $p = 0.014$), 10 ($\chi^2 = 8.41$, $p = 0.004$), and 100 μ M ($\chi^2 = 25.7$, $p < 0.001$) morphine compared with morphine alone. A similar observation had been made when compared with disulfiram alone. Of note, the Bliss synergy score of 22.92 ($p = 0.029$) suggested a synergistic interaction (Fig. S1). Disulfiram (0.1 μ M) also synergistically increased larvae mortality when combined with morphine (Tab. S1).

Table 1. The effect of (DSF, 0.1μM), morphine (MRF, 0.1-100μM), and their combination (MRF+DSF) on swim bladder inflation in zebrafish larvae at 118hpf. Results are presented as % of larvae with properly inflated swim bladders. The viability of 0.5% DMSO control was set to 100%

Treatment	Conc. (μM)	Sample size (n)	Swim bladder inflation (%)
0.5% DMSO	0	70	100
DSF	0.1	104	76.9 #
MRF	0.1	139	88.9
MRF	1	134	88.1
MRF	10	116	86.1 #
MRF	100	136	78.5
MRF0.1 + DSF0.1	-	37	60.3
MRF1 + DSF0.1	-	20	37.2 * \$\$\$
MRF10 + DSF0.1	-	36	32.5 * \$\$\$
MRF100 + DSF0.1	-	18	41.0 *** \$\$\$

*p<0.05; **p<0.01; ***p<0.001 (vs. MRF); #p<0.05 (vs. 0.5% DMSO control);

\$\$\$p<0.001 (vs. DSF).

Pearson's chi-square test. MRF-morphine, DSF-disulfiram.

Late swim bladder marker expression in zebrafish larvae exposed to disulfiram and morphine

The two-way ANOVA analysis of *acta2* gene expression indicated a significant interaction between swim bladder status (inflated vs. non-inflated) and treatment: [(F_{1,24} = 14.9, p = 0.0087)]. Likewise, a similar association was detected for the *anxa5a* gene [(F_{1,24} = 16.6, p = 0.00043)] (Fig. 4). The *acta2* gene was upregulated by 58.3% in zebrafish larvae with inflated swim bladders when compared with controls

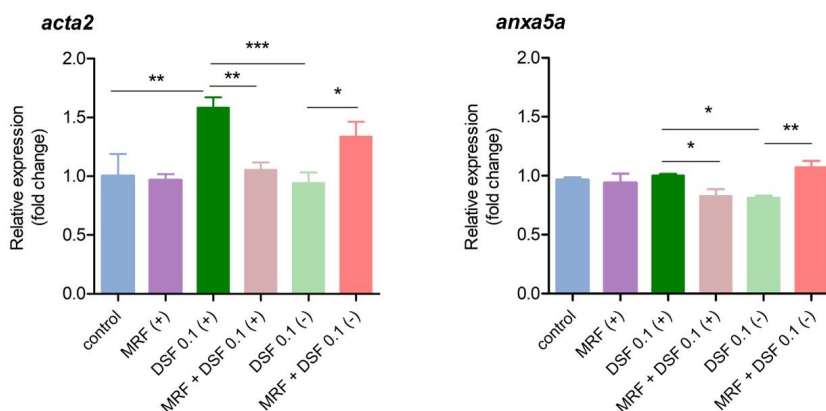


Fig. 4. mRNA levels of late mesenchymal (*acta2*) and mesothelial (*anxa5a*) swim bladder development markers in zebrafish larvae treated for 117hpf with disulfiram (DSF, 0.1μM), morphine (MRF, 10μM), and their combination (MRF + DSF) (n=4). (+) – larvae with correct swim bladder inflation, (-) – larvae with impaired swim bladder inflation. *p<0.05, **p<0.01; ***p<0.001 (two-way ANOVA, Duncan's test).

($p < 0.01$), whereas its expression remained unchanged in larvae with uninflated swim bladders. However, when $0.1 \mu\text{M}$ disulfiram was combined with morphine, *acta2* was upregulated in comparison with disulfiram alone. In contrast, in larvae with inflated swim bladders treated with the combination of morphine and disulfiram, the mRNA levels of the *acta2* gene were decreased ($p < 0.01$). Morphine alone did not affect *acta2* or *anxa5a* gene expression, nor did it significantly affect swim bladder inflation compared with controls (Tab. S1). Disulfiram treatment resulted in a small yet significant increase in *anxa5a* mRNA expression in larvae with intact swim bladders when compared to those with uninflated swim bladders ($p < 0.05$).

Similarly, as with the *acta2* gene expression, the addition of morphine significantly decreased *anxa5a* expression in larvae showing proper swim bladder inflation by 17.2% ($p < 0.05$). Again, morphine upregulated *anxa5a* gene expression in zebrafish with impaired swim bladder inflation by 31.5% ($p < 0.01$).

The developmental defects in swim bladder inflation caused by targeting the Hh signaling pathway includes heavy metals, insulation materials, and many popular drugs [Ali *et al.* 2014, Gao *et al.* 2023]. Morphine and disulfiram, a combination documented to effectively suppress morphine-induced pain hypersensitivity and withdrawal in experimental studies, was also recently shown to suppress swim bladder inflation in zebrafish larvae [Zelazewska *et al.* 2025b]. Thus far, previous investigations have provided evidence that this disruption following a 24-48h exposure of zebrafish embryos to morphine ($3\text{--}30 \mu\text{M}$) is possibly a product of drug-induced impairment of locomotor activity. Notably, motor impairment can negatively affect the high-powered swim-up movements to the air-water interface intended for gulping air, which is crucial for swim bladder inflation [Sales Cadena *et al.* 2021]. The importance of this behavior was highlighted in zebrafish *sox2*^{-/-} mutants, where the failure of swim bladder inflation was directly attributed to the abnormalities in motoneuron development [Cao *et al.* 2023]. However, our previous investigation showed no alterations in swimming patterns in disulfiram-morphine treated larvae with uninflated swim bladders. Only one report suggests that morphine could affect swim bladder morphogenesis by targeting the Shha pathway. An upregulation of Shha and the enhancement of its downstream effector expression—Ptch1 and Smo—was discovered in the spinal cord and dorsal root ganglia of adult CD-1 mice [Liu *et al.* 2018]. Interestingly, available data suggests a causative relationship between enhanced Shha signaling and the development of opioid-induced hyperalgesia, a conservative mechanism also present in the fruit fly [Babcock *et al.* 2011]. However, there are no documented reports showing the effects of subchronic exposure to morphine and disulfiram, alone or in combination, on the Hh pathway gene expression in developing zebrafish embryos in relation to swim bladder inflation.

As opposed to the aforementioned studies that were conducted in adult subjects, our investigation has shown that a 54h morphine treatment strongly decreased *shha* and *ptch1* mRNA levels, while failing to affect *ihha* expression. On the other hand, the mRNA levels of *ptch2* and *smo*, as well as of all the swim bladder markers analyzed

- *sox2*, *fgf10a* and *pbx1a* - were elevated 1.5- to 3-fold. Of note, morphine treatment only had a minor inhibitory effect on swim bladder inflation despite resulting in a substantial downregulation of the *shha* gene. It is possible that this triggered the local epithelial *ihha* and mesenchymal *ptch2* and *smo* upregulation to drive regional swim bladder morphogenesis and maintain tissue crosstalk despite systemic *shha* suppression. The importance of *ihha* was additionally corroborated by a significant synergistic detrimental effect of disulfiram on swim bladder inflation in morphine-treated larvae, where *ihha* expression was further decreased compared with both morphine and disulfiram alone.

An important role of *Ptch2* in *Shha* signaling in swim bladder and esophagus morphogenesis at early developmental stages had been reported before in the developing chick neural tube *Ptch1*^{-/-} mutant cells [Alfaro *et al.* 2014]. Another line of evidence suggests that zebrafish epithelial *Ihha*-dependent signaling is essential for the swim bladder mesenchymal layer formation as well as guiding the migration and differentiation of neural progenitors. Additionally, it has been suggested that abnormalities in mesenchymal layer proliferation seen in *ihha*^{-/-} zebrafish mutants are more pronounced in areas adjacent to *fgf10a* expressing regions, indicating that *Ihha* signaling is crucial for *Fgf10a*-mediated reciprocal relay of information back to the epithelium during swim bladder formation [Korzh *et al.* 2011]. The expression of *fgf10a* in morphine-treated zebrafish was increased almost 2-fold, implying that *Ihha* signaling could successfully compensate for morphine-induced deficiency in *shha* and *ptch1* expression.

The importance of *Ihha* signaling in swim bladder development is further strengthened by our observations that a decrease in *ihha*, *ptch2*, and *smo* mRNA levels induced by disulfiram was associated with a 23.1% decrease in the proportion of zebrafish with proper swim bladder inflation. Moreover, disulfiram had the ability to reverse morphine-upregulated expression of *ptch2* and *smo* in a synergistic fashion, causing the proportion of swim bladder inflation to drop from 86.1 to 32.5%. Our results showing a suppressive effect of disulfiram on the upstream Hh pathway genes complement previously-collected data demonstrating the inhibition of the Hh pathway downstream transcription factors *Gli1* and *Gli2* by disulfiram in multiple myeloma stem cells (MMSCs) [Jin *et al.* 2018]. Interestingly, disulfiram, as opposed to morphine, upregulated the expression of *acta2*, a late swim bladder smooth muscle marker. According to an earlier report, the downregulation of *acta2* does not seem to affect swim bladder inflation when zebrafish were exposed to iopanoic acid during the budding and pre-inflation phases that fall between 0-60hpf [Van Dingenen *et al.* 2023]. However, in our study, when disulfiram treatment was extended to the inflation phase (60-118hpf) a clear upregulation of *acta2* was noted, but only in larvae with inflated swim bladders. This could suggest a compensatory mechanism triggered by disulfiram alone. However, the mechanistic explanation for this phenomenon still needs to be evaluated.

Our previous investigation revealed an unexpected potentiation of disulfiram toxicity in a HepG2 cell line along with increasing concentrations of morphine

[Zelazewska *et al.* 2025c]. In zebrafish larvae treated with 0.1 μ M of disulfiram combined with morphine, a considerable 58% decrease in swim bladder inflation was observed when compared with disulfiram alone. At the same time, further downregulation of the upstream Hh pathway genes was also documented. Namely, the *ihha* and *shha* mRNA levels were substantially decreased while the expression of swim bladder markers - *sox2* and *fgf10a* - was elevated. Under the conditions of *ihha* and *shha* downregulation, the cells of the epithelial and mesenchymal layer cannot properly organize. In the loss-of-function *Shha* (*syu^{t4}*) and *Ihha* zebrafish morphants, it has been revealed that the presence of either *Shha* or *Ihha* alone is insufficient for swim bladder growth. The role of *Shha* is particularly important, because as evidenced in the *syu^{t4}* morphant, both the epithelial bud and the mesenchyme show a more severe lack of proper organization in comparison to the *Ihha* morphant [Winata *et al.* 2009b]. Similar observations were made in *Shha* mutant mice, where epithelial branching driven by Hh-dependent reciprocal signals from the bronchial mesenchyme was disrupted and resulted in pulmonary hypoplasia [Pepicelli *et al.* 1998b]. Therefore, a possible explanation for *sox2* and *fgf10a* gene upregulation could be related to the triggering a compensatory mechanism directed at increasing epithelial and mesenchymal layer proliferation to compensate for the downregulation of Hh signaling. A deficiency in Hh signaling also disrupts the organization and proliferation of the outer mesothelium. The appropriate structuring of the outer mesothelium around the mesenchyme appears to require mesenchyme differentiation into smooth muscle tissue via the expression of the *anxa5a* marker. Our investigation showed that in zebrafish larvae exposed to the disulfiram-morphine combination, *acta2* and *anxa5a* gene expression was upregulated only in larvae with uninflated swim bladders, further implying the existence of a compensatory mechanism in the conditions of considerable *ihha* and *shha* downregulation.

Despite disulfiram being investigated as a potential treatment option for the prevention of morphine-induced hyperalgesia, its use could be associated with a higher risk of teratogenicity. Our study has provided first-time evidence that the disruption in swim bladder inflation in zebrafish following combined disulfiram and morphine treatment might be associated with *shha* and *ihha* downregulation. However, it must be acknowledged that the Hh pathway gene expression analyses were performed using whole-embryo RNA rather than RNA isolated specifically from the swim bladder layers due to technical limitations. Therefore, the observed changes in transcript levels additionally reflect contributions from multiple embryonic tissues and cannot be attributed exclusively to the swim bladder alone. Nevertheless, these transcriptional changes occurred in parallel with a robust and reproducible impairment of swim bladder inflation in exposed embryos. Thus, while our qPCR data does not specifically demonstrate swim bladder-specific alterations in Hh pathway gene expression, the concordance between altered whole-embryo Hh signaling and the swim bladder inflation phenotype supports a potential role for disrupted Hh signaling in the developmental defects observed in this organ. Future studies employing

tissue-specific approaches will be necessary to determine the extent to which these transcriptional changes occur within the swim bladder itself. Additionally, it has yet to be determined whether the inhibition of the upstream Hh pathway could also negatively affect pulmonary morphogenesis in mammals.

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Supplementary data

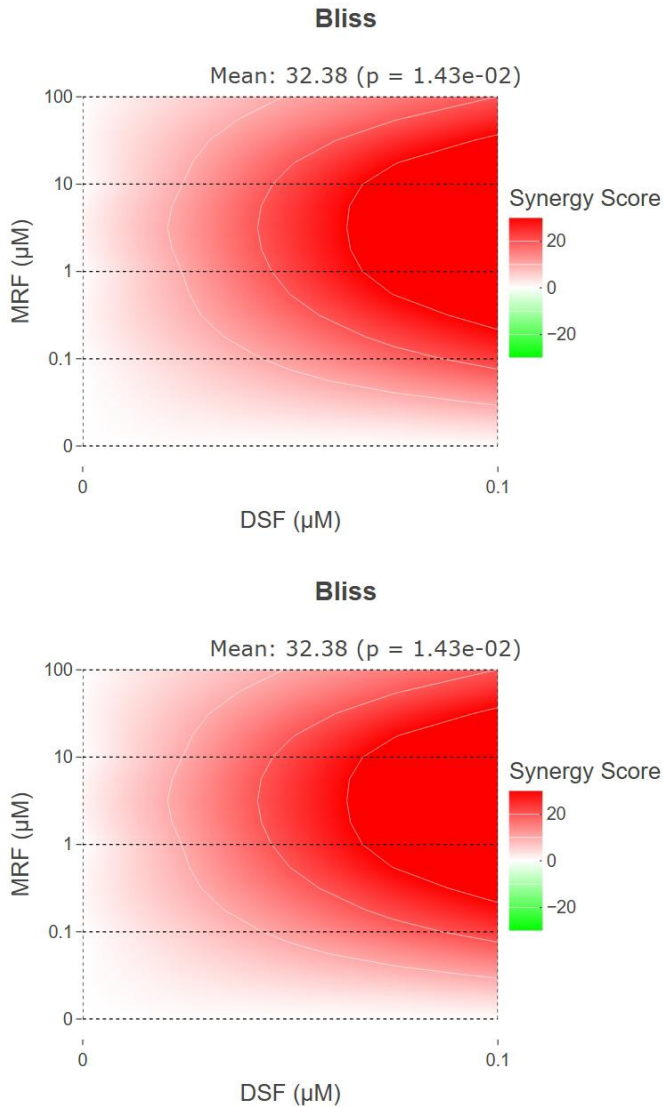


Fig. S1. Synergy heatmap of the effect of the disulfiram-morphine combination on swim bladder inflation (top) and overall survival (bottom). Synergy scores were calculated using the Bliss Independence model. Shades of red indicate the type and strength of the synergistic interaction. The Bliss Synergy Score (BSS) score < -10 indicates antagonism; Values between $-10 < \text{BSS} < 10$ indicate an additive effect. Values $\text{BSS} > 10$ indicate synergism.

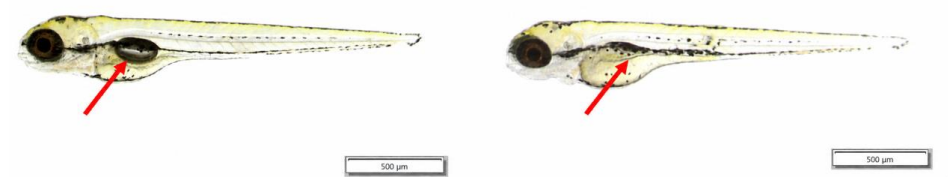


Fig. S2. A representative image of larvae with an inflated (left) and uninflated (right) swim bladders. Left image: control larva treated with 0.5% DMSO. Right image: larva treated with a disulfiram (0.1 µM) and morphine (10 µM) combination. The red arrow points to the phenotype being analyzed ie. inflated or uninflated swim bladder. Scale bar 500 µM. An AI tool was used to remove distortions in the background.

Table S1. Cumulative number of dead zebrafish larvae exposed to disulfiram (DSF, 0.1 µM), morphine (MRF, 0.1-100 µM) and their combination (MRF+DSF) from 1 to 118 hpf. Results are presented as % of dead larvae. The 0.5% DMSO control was set to 0%

Treatment	Conc. (µM)	Sample Size (n)	Dead (%)
0.5% DMSO	0	34	0
DSF	0.1	77	26.1
MRF	0.1	62	16.8
MRF	1	53	19.7
MRF	10	42	24.1
MRF	100	69	38.6 #
MRF0.1 + DSF0.1	-	52	39.7 \$
MRF1 + DSF0.1	-	52	62.7***\$\$\$
MRF10 + DSF0.1	-	61	67.5 ***\$\$\$
MRF100 + DSF0.1	-	29	58.7 \$\$

*p<0.05; **p<0.01 ***p<0.001 (vs. MRF); #p<0.05 (vs. 0.5% DMSO control); \$\$\$p<0.001 (vs. DSF).
Pearson's chi-square test. MRF-morphine, DSF-disulfiram.

Table S2. Primer sequences for zebrafish swim bladder genes

Gene	Forward primer (5'→3')	Reverse primer (5'→3')	Amplicon size (bp)	Accession no.
<i>shha</i>	CGAGTCCAAAGCCACATTC	GGACAAAAAGGAGGTGAGCG	299	NM_131063.3
<i>ihha</i>	TGGTGCGAAGACTGAGCTTGA	TAAACGTCCGTGCTGTCCTG	163	NM_001034993.2
<i>pchl</i>	GATTGGGACTCTGCTCGCTT	GGGATAAGGGCGGCCATAAA	241	NM_001305542.1
<i>pchl2</i>	GCGCCATCTCCACTCTTTTG	CTGATGTGATGCCTTGGGA	278	NM_130988.2
<i>Sno</i>	TTGGTCTCTGCTTTGGAGC	TGCCCCATTGATCTTGGTCT	129	NM_131027.2
<i>sox2</i>	CTCGGAAACAACACAGAAAA	TCGCTCTCGGACAGAAATT	171	NM_213118.1
<i>fgf10a</i>	ATCGTTGCCATCAAGGGGATT	AAATGTTCCCTTTGGTGGGT	293	NM_182870.2
<i>pbx1a</i>	CGCCGAACACTCAGACTACA	GAAGTTCCTCCTGCTCGAG	280	XM_017358962.3
18S [Gao et al. 2024]	TCGCTAGTTGGCATCGTTTATG	CGGAGGTCGAAGACGATCA	162	BX296557
<i>Elf1a</i> [McCurley et al. 2008]	CTTCTCAGGCTGACTGTGC	CCGCTAGCATTACCCCTCC	358	AY422992
<i>anxa5a</i>	GTGATACTCGTACAGGCGGG	TGAACTTGCTCTGCTCCGTG	118	NM_001113582.1
<i>acta2</i>	GAGCGGTGTTCCCTTCCATA	GATTCCCCCTCTTGCTTTGCG	110	NM_212620.2